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Correlation Between Histochemically Assessed Fiber Type Distribution and Isomyosin and Myosin Heavy Chain Content in Porcine Skeletal Muscles[1]

G. Bee*,[2], M. B. Solomon*,[3], S. M. Czerwinski†, C. Long‡, and V. G. Pursel‡

*USDA, ARS, Meat Science Laboratory, †Growth Biology Laboratory, and ‡Gene Evaluation and Mapping Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705-2350

ABSTRACT: Highly sensitive enzyme assays developed to differentiate skeletal muscle fibers allow the recognition of three main fiber types: slow-twitch oxidative (SO), fast-twitch oxidative glycolytic (FOG), and fast-twitch glycolytic (FG). Myosin, the predominant contractile protein in mammalian skeletal muscle, can be separated based on the electrophoretic mobility under nondissociating conditions into SM_2, SM_1, IM, FM_3, and FM_2 isoforms, or under dissociating conditions into myosin heavy chain (MHC) I, IIb, IIx/d, and IIa. The purpose of the present study was to determine whether the histochemical method of differentiation of fiber types is consistent with the electrophoretically identified isomyosin and MHC isoforms. These comparisons were made using serratus ventralis (SV), gluteus medius (GM), and longissimus muscles (LM) from 13 pigs. Two calculation methods for the histochemical assessed fiber type distribution were adopted. The first method incorporated the number of fibers counted for each fiber type and calculated a percentage of the total fiber number (fiber number percentage: FNP). The second method expressed the cross-sectional area of each fiber type as a percentage of the total fiber area measured per muscle (fiber area percentage: FAP).

Independent of the calculation methods, correlation analyses revealed in all muscles a strong relation between SO fibers, the slow isomyosin (SM_1 and SM_2), and MHCI, as well as between the FG fibers, the fast isomyosin (FM_3 and FM_2), and MHCIIx/b content ($P < .05$). There were no correlations between FOG fiber population assessed by histochemical analysis and intermediate isoform (IM) or MHCIIa content. The present results did not provide conclusive evidence as to which of the calculation methods (FNP or FAP) was more closely related to myosin composition of skeletal muscles. Despite some incompatibility between the methods, the present study shows that histochemical as well as electrophoretic analyses yielded important information about the composition of porcine skeletal muscle. The combination of the two methods may be essential to accurately characterize porcine skeletal muscles.

Key Words: Pigs, Histochemistry, Myosins, Skeletal Muscle

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Introduction

The development (Solomon and Dunn, 1988) of highly sensitive enzyme assays permits the identification and evaluation of individual fiber types in skeletal muscle. These techniques couple the detection of succinate dehydrogenase (**SDH**) with myofibrillar ATPase (**mATPase**) activity, and allow for differentiation of slow-twitch oxidative (**SO**), fast-twitch oxidative glycolytic (**FOG**), and fast-twitch glycolytic (**FG**) fiber types.

Myosin is the most abundant contractile molecule in mammalian skeletal muscles. This polymorphic molecule exists in different isoforms. Isomyosins can be separated based on their electrophoretic mobility under nondissociating conditions into three slowly migrating forms (SM1, SM2, and IM), typical for slow-twitch muscles, and three rapidly migrating forms (FM3, FM2, and FM1), typical for fast-twitch muscles (Marechal et al., 1989). Under dissociating conditions, myosin can be separated into three myosin heavy chain (**MHC**) isoforms using SDS-PAGE. The MHCI is expressed in type I fibers, MHCIIa in type IIA, and MHCIIb in type IIB (Staron and Pette, 1987).

[1]Mention of trade names or companies does not constitute an implied warranty or endorsement by the USDA or the authors.

[2]Visiting Scientist from Institute of Animal Science, Nutrition Biology, ETH Zürich, Switzerland.

[3]To whom correspondence should be addressed.

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Our objective for the present study was to determine whether a histochemical method of differentiation of fiber types provides results consistent with the electrophoretically identified isomyosin forms under nondissociating conditions, and MHC isoforms under dissociating conditions. These comparisons were made using serratus ventralis, gluteus medius, and longissimus muscles from 13 pigs.

Materials and Methods

Animals

Thirteen intact male pigs from different litters were used in this study. Pigs were weaned at 3 to 4 wk of age and had access to a corn-soybean meal diet containing 18% crude protein at 90% free access until they reached 120 kg of body weight.

Tissue Sampling

Pigs were electrically stunned, exsanguinated, and eviscerated according to USDA regulations. Within 1 h after exsanguination, portions of muscles were removed from the left side of each carcass. Muscles included the longissimus (**LM**) and serratus ventralis (**SV**) from the torso, and the hindlimb muscle, gluteus medius (**GM**). Muscle samples were removed from the central region of the SV and GM and from the anterior of the 12th rib location for the LM. One piece (approximately $1 \times 1 \times 3$ cm) of each muscle was fixed immediately on a labeled flat stick and frozen in liquid nitrogen. The samples were removed from liquid nitrogen, wrapped in aluminum foil, and stored at –70°C until histochemical and electrophoretic analyses were performed.

Histochemical Methods

Frozen muscle samples were equilibrated to –25°C, and a small section removed from the stick and trimmed to facilitate transverse sectioning. Samples were mounted on cryostat chucks with a few drops of tissue freezing medium (Triangle Biomedical Science, Durham, NC). Six-micrometer-thick sections were cut using a Cryostat 2800 Frigocut-E (Reichert-Jung, Cambridge Instruments, New York). Sections were mounted on glass microscope slides and were allowed to air-dry for 30 min. Sections were treated with the combination acid myofibrillar ATPase and SDH staining procedure described by Solomon and Dunn (1988). Stained sections were observed at 20× with a Zeiss Confocal microscope (Carl Zeiss, New York) in transmitted light mode. Muscle fibers were classified as SO, FOG, and FG based on the stain reaction according to Peter et al. (1972). Four images at different locations within each slide of each muscle sample were captured on a computer and analyzed with the Kontron 300 image analysis software (Kontron Elektronik GmbH, Germany). Muscle fibers not representing intact cross-sections at the edges of the images were not included in the calculations. Fiber area was expressed as square micrometers. Two calculation methods for the fiber type distribution were adopted. The first method incorporated the number of fibers counted for each fiber type and calculated a percentage of the total fiber number counted (fiber number percentage: **FNP**). The second method expressed the cross-sectional area (**CSA**) of each fiber type reported as a percentage of the total fiber area measured per muscle (fiber area percentage: **FAP**).

Myosin Extraction

Adjacent to the samples taken for histochemical analysis, approximately 100 mg of muscle tissue was removed. For preparation of myosin extracts, muscle samples were homogenized 1:7 (wt/vol) in 40 m*M* NaCl, 3 m*M* NaH_2PO_4, and 3 m*M* Na_2HPO_4. The homogenates were centrifuged at $1,500 \times g$ for 10 min, resuspended in homogenizing buffer, and centrifuged at $1,500 \times g$ for 10 min. Supernatant was discarded and pellets were resuspended 1:1.5 (wt/vol) in extraction buffer (.1 *M* $Na_4P_2O_7$, 5 m*M* EGTA, 5 m*M* DTT, and leupeptin (5 μg/mL), incubated for 1 h on ice, and centrifuged at $40,000 \times g$ for 30 min.

Native Gel Electrophoresis of Isomyosins

Native gel electrophoresis was performed following the protocol of Reisner et al. (1976). Briefly, the native gel electrophoresis was carried out in tube gels (5-mm i.d., 7.5 cm long) at 4°C for 21 h at 90 V (Hoh et al., 1976). Myosin extract was mixed with loading buffer (1:40, vol/vol) containing 1 m*M* EDTA, 5 m*M* cysteine, .0004% bromphenol blue, 50% (vol/vol) glycerol, and 30 m*M* sodium pyrophosphate (pH 8.6). Gels were stained with coomassie brilliant G-250 (.04% wt/vol in 3.5% vol/vol perchloric acid). Each sample was electrophoretically typed in duplicate.

Myosin Heavy Chain Analysis

Myosin heavy chain analysis was performed on the muscle samples using SDS-PAGE. The protocol was based on the procedure described by Talmadge and Roy (1993). Briefly, the supernatant from the myosin extraction was heated for 3 min at 100°C in a lysine running buffer (1:2 vol/vol) containing 10% vol/vol glycerol, 5% vol/vol 2-mercaptoethanol, and 2.3% wt/vol SDS in 62.5 mmol/L Tris/HCl buffer (pH 6.8) (Laemmli, 1970). The extracts (3 to 7.5 μL) were loaded on 8% SDS-polyacrlyamide gels (4.5 cm long) with a 4% stacking gel (1.5 cm long), run for 20 to 22 h at 70 V in a Bio-Rad (Hercules, CA) minigel apparatus. Subsequently, the gels were stained with coomassie blue stain solution (50% vol/vol methanol, 10% vol/vol glacial acetic acid, and .06% wt/vol

coomassie blue R250) for 2 h at 45°C and destained in methanol (10% vol/vol) and acetic acid glacial (10% vol/vol) until defined bands could be detected.

The relative proportions of the different isomyosin and MHC isoforms were quantified with densitometry (Ultrascan XL laser densitometer, LKB-Pharmacia, Piscataway, NJ). Adult rat skeletal muscle samples (soleus and gastrocnemius muscle) were used for comparisons. Comparisons between rat and porcine tissues extracts were made by measuring relative mobilities of each isomyosin and MHC band.

Statistical Analysis

Descriptive statistics were performed using SAS PC software (SAS, 1996) to calculate means and SEM for all variables. Differences among fiber types for the CSA and differences between the fiber distribution calculated as either FNP or FAP were examined by a one-way ANOVA. Correlation analysis was generated to compare FNP, FAP, native isomyosin, and MHC concentration. Statistical significance was accepted at $P < .05$.

Results and Discussion

Fiber Types

The muscle samples chosen intentionally exhibited varying amounts of the three fiber types (Table 1). According to their fiber type distribution, the GM and LM were fast-twitch muscles. In the four randomly selected locations within each slide the muscles had, on average, more FG (GM = 144, LM = 158) and fewer FOG (GM = 40, LM = 36) and SO fibers (GM = 61, LM = 36). The CSA of FG fibers was greater ($P < .05$) than that for the SO and FOG fibers in the GM and LM (Table 1). The percentage of FG fibers calculated as FAP was higher ($P < .05$) in the GM and LM; the percentage of FOG was lower ($P < .05$) than that calculated with the FNP method.

The SV muscles exhibited a heterogeneous composition. The average number of measured fibers within each muscle amounted to 66, 84, and 95 for the SO, FOG, and FG fibers, respectively. The CSA of FOG fibers was smaller than that of the SO and FG fibers in the SV (Table 1). The percentage of FOG fibers calculated as FAP was lower ($P < .05$) than that calculated with the FNP method; no differences existed for the SO and FG fiber types between the two methods of calculation.

The automated analysis method applied in this study allowed the measurement of number and size of a large area (between 700 and 800 mm^2) within each muscle section, and, therefore, two calculation methods to express the fiber type distribution were compared. The results provide clear evidence that the fiber distribution within each muscle is strongly affected by the method of calculation. In contrast to the FNP method, the FAP method consists not only of the number of fibers but also the mean CSA of each fiber type. Atrophy of selected muscle fibers, for instance, induces changes in the contractile properties such as shortening velocity and fatigue resistance and alters the myosin heavy chain expression (Van Balkom et al., 1997). Therefore, it may be more accurate to express the fiber distribution as FAP than FNP, in order to account for the physiological properties of the muscle.

Table 1. Cross-sectional area (CSA), FNP,[a] and FAP[b] of the three fiber types identified by the combination stain procedure in three muscles of pigs[c]

	Fiber type		
Muscle	SO[d]	FOG[e]	FG[f]
Gluteus medius (n = 13)			
CSA, μm^2	2650 (187)[g]	2522 (208)[g]	4027 (227)[h]
FNP, %	16.3 (2.30)	25.2 (1.74)	58.5 (2.76)
FAP, %	12.4 (1.62)	18.8 (1.88)*	68.8 (2.42)*
Longissimus muscle (n = 13)			
CSA, μm^2	3118 (318)[g]	2601 (194)[g]	4049 (230)[h]
FNP, %	12.2 (1.62)	16.5 (1.18)	71.3 (1.74)
FAP, %	9.5 (1.25)	11.9 (1.08)*	78.6 (1.50)*
Serratus ventralis (n = 13)			
CSA, μm^2	3097 (236)[h]	2135 (156)[g]	3432 (167)[h]
FNP, %	34.0 (2.29)	27.2 (1.65)	38.9 (2.40)
FAP, %	35.3 (2.24)	19.6 (1.38)*	45.1 (2.48)

[a]FNP = fiber number percentage is the ratio of the number of counted fibers of each fiber type to the total counted fiber number.
[b]FAP = fiber area percentage is the ratio of the total cross-sectional area of each fiber type to the total measured fiber area.
[c]Values are mean (SEM).
[d]SO = slow-twitch oxidative.
[e]FOG = fast-twitch oxidative glycolytic.
[f]FG = fast-twitch glycolytic.
[g,h]Cross-sectional areas across fiber type differs ($P < .05$).
*$P < .05$ compared with the same fiber type calculated as FNP.

Native Isomyosin Distribution

The GM, LM, and SV muscles of pigs predominantly contain protein bands that correspond to rat slow (SM_2 and SM_1), intermediate (IM), and fast isomyosin (FM_3 and FM_2) (Figure 1). Based on the data presented and on a previous study on porcine longissimus muscle (Czerwinski and Martin, 1994), pig skeletal muscle myosin isoforms seem to have the same electrophoretic mobilities as myosin isoforms of rat. According to Marechal et al. (1989) and d'Albis et al. (1979), the difference in the electrophoretic mobility seems to be determined either by the content of myosin heavy chain or myosin light chain or from both.

The fast isomyosin forms FM_3 and FM_2 accounted for more than 50% and the IM isoform for 39 and 46%

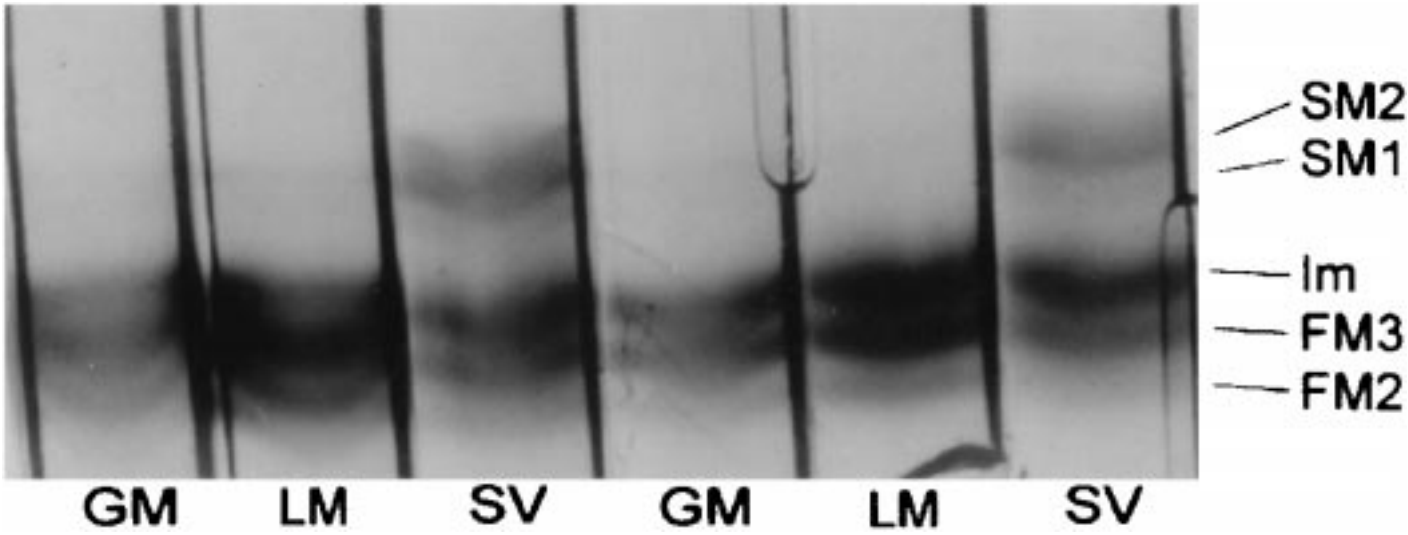

Figure 1. Gel electrophoresis in nondissociating conditions of myosin isozymes in gluteus medius (GM), longissimus dorsi (LM), and serratus ventralis (SV) muscles of pigs. SM_2 = slow isomyosin two; SM_1, slow isomyosin one; IM, intermediate isomyosin; FM_3, fast isomyosin three; FM_2, fast isomyosin two.

of the total isomyosin in the GM and LM (Table 2). Several studies identified the FM_3 and FM_2 isomyosins in muscles containing predominantly IIB fibers, whereas IM isomyosin was observed in muscles containing the IIA fibers (Fitzsimons and Hoh, 1983; d'Albis et al., 1986; Gregory et al., 1986; Tsika et al., 1987; Marechal et al., 1989).

The isomyosin distribution of the SV muscle was characterized by an almost equal amount of slow, intermediate, and fast isomyosin. In all three muscles, FM_3 was the predominant fast isomyosin. In the GM muscles, both slow isoforms (SM_2 and SM_1) were nearly equally distributed, whereas in the SV, SM_2 was the major slow isoform. In the LM, only traces of slow isomyosin were detected. These findings are in agreement with studies of isomyosin patterns of fast-twitch, slow-twitch, and mixed skeletal muscles of small mammals (Marechal et al., 1989; Termin and Pette, 1991).

Myosin Heavy Chain Distribution

Electrophoresis of MHC from the GM, LM, and SV muscles indicated three resolvable bands (Figure 2). The identities of the three protein bands were compared to the mobility of MHC of rat skeletal muscles. Two of the MHC isoforms of the pig muscles comigrated with type I and IIa MHC of rat soleus and gastrocnemius muscle. The third isoform had a mobility between type IIb and IIa of rat gastrocnemius. Accordingly, the bands were designated as MHCI, MHCIIx or MHCIIb, and MHCIIa. In the GM and LM muscles, the type IIx/b and IIa isoform were the most abundant, with small amounts of MHCI (Table 3). In the SV, the predominant isoform was IIa, with smaller amounts of type I and IIx/b.

Single fiber studies of rat, rabbit, and human skeletal muscles revealed that each main fiber type contained exclusively one MHC isoform (Staron and

Table 2. Native isomyosin distribution in three muscles in pigs[a]

	Native isomyosin distribution, %						
Muscle	SM_2	SM_1	SM[b]	IM	FM_3	FM_2	FM[c]
Gluteus medius	3.4 (.28)	4.4 (.32)	7.9 (.55)	39.2 (1.53)	40.8 (.93)	12.2 (1.13)	53.0 (1.84)
Longissimus	1.4 (.44)	1.6 (.42)	3.0 (.81)	45.7 (1.83)	40.8 (1.21)	10.5 (1.04)	51.3 (1.90)
Serratus ventralis	21.5 (1.95)	10.2 (.60)	31.7 (2.31)	35.7 (1.43)	26.8 (1.34)	5.8 (.57)	32.6 (1.70)

[a]Values are mean (SEM).
[b]SM = sum of SM_1 and SM_2.
[c]FM = sum of FM_3 and FM_2.

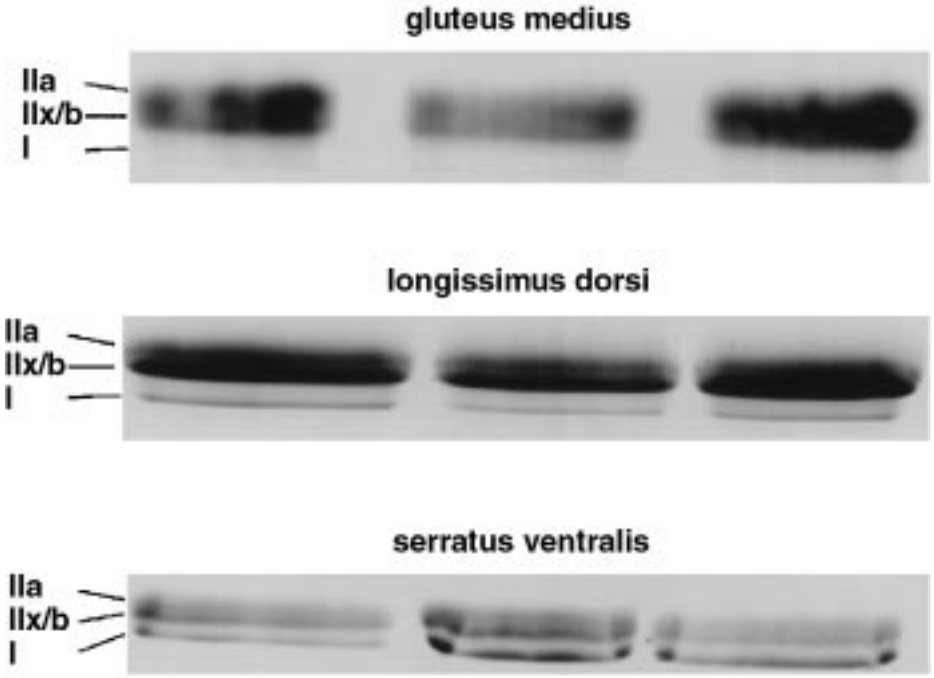

Figure 2. Eight percent SDS-PAGE of myosin heavy chain (MHC) in gluteus medius, longissimus, and serratus ventralis muscles of pigs. The MHC isoforms are identified as types I, IIx/b, and IIa.

Pette, 1986; Termin et al., 1989; Staron, 1991; Fry et al., 1994). Their results showed that MHCI, MHCIIa, and MHCIIb were present as unique isoforms in histochemically defined fiber types I, IIA, and IIB, respectively. In our study, the results of the MHC distribution differed from the results of the fiber type profile. The relative amount of FOG fibers determined by the simultaneous combination stain technique was lower and the amount of FG fibers was higher compared to the corresponding MHC isoforms (Tables 1 and 2). The discrepancy between the electrophoretic and histochemical data is especially evident in the fast-twitch muscle fibers and the fast MHC isoforms.

Recent investigations established that in skeletal muscles of small and large mammals, beside the three main fiber types, a variety of hybrid fibers exist (Staron and Pette, 1986; Holly and Wass, 1989; Gorza, 1990; Staron, 1991; Staron and Hikida, 1992; Schiaffino and Reggiani, 1994; Talmadge et al., 1995). The hybrid fibers reveal intermediate histochemical as well as immunohistochemical stain intensities between type I and IIA (classified as IC, IIC) as well as between type IIA and IIB (classified as IIAB) and express the different MHC isoforms in varying amounts.

The histochemical technique used in this study allows the differentiation of three main fiber types, combining two enzyme staining procedures (acid mATPase at pH 4.3, with SDH). A differentiation of hybrid fibers is not possible using this histochemical technique; therefore, in the present study some of the three main fiber types may have been misclassified, which could explain the discrepancy between the histochemical and electrophoretic data.

The simple relationship between muscle fiber types and MHC composition demonstrated in the single fiber studies had been complicated by the findings of coexpression of two or more MHC isoforms in some fibers. Danieli-Betto et al. (1986) reported that in rats some type I, type IIA, and type IIB fibers showed—in addition to the corresponding MHCI, MHCIIa, and MHCIIb—the presence of another MHC. In type I and IIB fibers the extra MHC was always the type IIA, and these fibers contained either MHCI or MHCIIb. Their results indicated that coexistence of different MHC isoforms was more frequent in type II fibers than in type I. Therefore, the discrepancy between the histochemical and electrophoretic data may be explained by the fact that in porcine muscles a certain number of SO and FG fibers coexpress the

Table 3. Myosin heavy chain (MHC) distribution in three muscles in pigs[a]

Muscle	MHCI	MHCIIa	MHCIIx/b
Gluteus medius	2.8 (.23)	58.6 (1.53)	38.6 (1.52)
Longissimus	5.8 (.74)	47.2 (2.00)	47.1 (2.21)
Serratus ventralis	31.9 (1.53)	42.7 (1.20)	25.4 (1.69)

[a]Values are mean (SEM).

Table 4. Correlation analysis between fiber type distribution, relative cross-sectional area (CSA), isomyosin pattern, and myosin heavy chain (MHC) distribution for all three muscles combined

Item	Histochemical classification:	SO[a]	FOG[b]	Fg[c]
	Isomyosin:	SM[d]	IM	FM[e]
	MHC:	I	IIa	IIx/b
FNP[f] vs FAP[g]		.95	.92	.96
Isomyosin distribution vs FNP		.86	–.44	.69
Isomyosin distribution vs FAP		.92	–.31*	.74
MHC vs FNP		.80	–.05*	.76
MHC vs FAP		.90	.02*	.75
MHC vs Isomyosin		.95	.16*	.73

[a]SO = slow-twitch oxidative.
[b]FOG = fast-twitch oxidative glycolytic.
[c]FG = fast-twitch glycolytic.
[d]SM = SM_1 + SM_2.
[e]FM = FM_1 + FM_2.
[f]FNP = fiber number percentage describes the ratio of the number of counted fibers of each fiber type to the total counted fiber number.
[g]FAP = fiber area percentage describes the ratio of the total CSA of each fiber type to the total measured fiber area.
*Correlation value not significant ($P > .05$).

MHCIIa isoform. Fibers coexpressing two or more MHC isoforms react histochemically as well as immunohistochemically according to the dominant isoform (Danieli-Betto et al., 1986; Klitgaard et al., 1990).

Histochemical and Electrophoretic lnterrelationships

Histochemically determined fiber distribution expressed as FNP or FAP for the three fiber types was compared to the isomyosin and MHC contents (Table 4). Correlation analyses revealed a strong relation between SO fibers, SM, and MHCI, and between FG fibers, FM, and MHCIIx/b content ($P < .05$). For SO fibers, the correlations between electrophoretic and histochemical data were higher if the fiber distribution was expressed as FAP than as FNP; for the FG fibers the correlations were similar. Population of FOG fibers assessed by histochemical analysis and IM and MHCIIa content showed no significant correlation.

The low negative correlation between FOG fibers and the IM isomyosin and MHCIIa content was unexpected. With the simultaneous combination stain technique, the FOG fibers are separated from the FG fibers according to their aerobic oxidative capacity by using the SDH activity as a marker (Solomon and Dunn, 1988). From our results, one can assume that the SDH activity is a poor indicator of IM isozyme and MHCIIa content. In a recent study, fiber SDH activity did not seem to be determined by the MHC isoform expression (Sieck et al., 1996). Therefore, it may not be suitable to compare the FOG fiber content with the MHC or isomyosin isoform. In addition, there is evidence that an incompatibility of the mATPase-based and metabolic enzyme-based classification may exist. Reichmann and Pette (1982) and White and Snow (1985) have shown that a pronounced overlap exists in SDH activity between type IIB and IIA fibers. Even though the mean value of SDH activity can be used to separate type IIA and IIB fibers in mammals, Pette and Staron (1990) reported that some type IIB fibers were as oxidative as IIA fibers, and some IIB fibers exhibited SDH activities as high as those found in the majority of the IIA fibers. This may be explained by the recently characterized fiber type, classified as IIX or IID (Baer and Pette, 1988; Schiaffino et al., 1989; Gorza, 1990). This fiber type seems to be characterized by an aerobic oxidative capacity between that of type IIA and of IIB fibers (Schiaffino and Reggiani, 1994). The IIX/IID fiber type is characterized by a specific MHC isoform, MHCIIx/IId (Schiaffino et al., 1989; Termin et al., 1989; Larsson et al., 1993). De Nardi et al. (1993) have shown that MHCIIx/IId is a product of a distinct gene and is not produced by posttranslational modification of other MHC genes.

Several investigators have demonstrated that the continuum of fiber types is difficult to quantify, and, therefore, correlation coefficients between histochemical and electrophoretic data may be low (Staron and Hikida, 1992; Fry et al., 1994; Rivero et al., 1996; Serrano et al., 1996). The correlations between isomyosin and MHC content in the present study revealed a significant positive relationship. Nevertheless, the correlation was higher for the slow-twitch isoforms than it was for the fast-twitch isoforms. The molecular weights of the three fast MHC are very similar. The resolution provided by 8% SDS-PAGE was not always satisfactory using this protocol, which was developed for rat skeletal muscles. Therefore, the

quantification of the MHCIIa and MHCIIx/b could be subject to some errors. However, the present results suggest that porcine skeletal muscles contain three distinct MHC isoforms. Two of the isoforms comigrated with type I and IIa MHC isoforms of rat skeletal muscle. The electrophoretic mobility of the third isoform was closer to type IIa than it was to type IIb MHC isoform of rat gastrocnemius. A possible explanation could be that this porcine MHC is a type IIb MHC isoform but with a higher molecular weight than type IIb MHC of rat. However, this MHC isoform could also be more similar to the rat type IIx. The isomyosin pattern did not clarify this question and therefore further investigation is needed to elucidate this point.

Implications

The present study shows that histochemical as well as electrophoretic analyses yield important information about the composition of porcine skeletal muscle. The relationship between the myosin composition and fiber type distribution of skeletal muscle is complicated by at least two factors. On the one hand, a continuum of fiber types exists; on the other hand, two or three myosin heavy chains can coexist in the same fiber type. Therefore, more effective histochemical or immunohistochemical methods are needed to determine the muscle fiber population. The present results did not provide conclusive evidence as to which calculation method (fiber number percentage or fiber area percentage) more closely related to myosin composition of skeletal muscles.

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